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Predominance of *afr2* and *ral* Fimbrial Genes Related to Those Encoding the K88 and CS31A Fimbrial Adhesins in Enteropathogenic *Escherichia coli* Isolates from Rabbits with Postweaning Diarrhea in Central Europe

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PCR tests designed in these studies identified three rabbit adhesive factor genes among 43 enteropathogenic *E. coli* (EPEC) strains: *afr1* (2 strains), the F4(K88)/CS31A-related *afr2* (10 strains), and *ral* (15 strains). Several EPEC strains (i.e., O153:H7 and O157:H2) lacked these genes but did adhere to HeLa cells and produced attaching and effacing lesions in rabbits.

Enteropathogenic *Escherichia coli* (EPEC) is an important cause of morbidity among infants in nonindustrialized countries and among young animals (32). It has been established that strains of *E. coli* that are highly pathogenic for weaned rabbits are EPEC (8, 27, 37, 38). EPEC typically do not produce known enterotoxins or Shiga toxins but damage the intestinal epithelial cells by effacing the microvilli and attaching intimately to the cell membrane. This leads to the characteristic "attaching and effacing" (AE) lesion and diarrhea (12, 25, 40). The ability of rabbit EPEC to produce AE lesions is due to the locus of enterocyte effacement (LEE) pathogenicity island inserted into the chromosome. LEE codes for the 94- to 97-kb outer membrane protein called intimin (Eae) and its translocated receptor (Tir) and for a type III secretion system which translocates the Tir protein into the host cell (46, 52). Variability of *eae* genes has led to the establishment of 10 different types (α, β, γ, δ, ε, η, ι, κ, θ, and ξ) (36, 51). In addition to the LEE, several colonization factors also have been found in rabbit EPEC. The adhesion to enterocytes and the colonization aptitude of the most intensively studied rabbit diarrheal *E. coli* (RDEC-1) were correlated to the presence of a plasmid-encoded fimbrial adhesin called adhesive factor/rabbit 1 (AF/R1) (13). Another fimbrial adhesin, adhesive factor/rabbit 2 (AF/R2), which confers on EPEC strains the ability to attach to rabbit enterocytes and to HeLa cells in a diffuse manner, is also associated with in vivo virulence (28). The *afr2* operon is located on the chromosome (17). A further adhesin, called Ral (for rabbit EPEC adherence locus), has also been identified, and its genetic determinant (*ral*) was located on a plasmid (1, 23). Both AF/R2 and Ral share homology with the F4(K88) and CS31A adhesins (1). Ral and intimin act as adhesion factors cooperatively, with Ral-mediated adhesion preceding that mediated by intimin (23). Another virulence gene involved in the capability of enterohemorrhagic *E. coli* and EPEC to adhere to mammalian cells in vitro and in repression of the host lymphocyte activation response is the *efa1/lifA* gene (22, 45). The gene *paa* (porcine attaching and effacing-associated gene), first reported to be present in porcine EPEC, was also shown to be present in rabbit *E. coli* (3, 4, 5). Finally, long polar fimbriae similar to those of enterohemorrhagic *E. coli* O157:H7 may also act cooperatively with rabbit-specific fimbriae in early steps of the adhesion of rabbit EPEC (33).

Epidemiological studies revealed that EPEC strains isolated from diarrhoeic weaned rabbits most frequently harbor the β type of *eae* (24, 36, 41). EPEC strains isolated in Belgium, The Netherlands (39), and the United States (12) belonged to serotype O15:K−H−, while rhamnose-negative O103:K−:H2 strains are predominant in France (11) and Spain (6, 7, 8, 24). In addition to Spain and France, strains of serogroup O103 were also detected in Belgium, Germany, Hungary, Italy, and the United States (18, 30, 32, 34, 37, 49). Studies in Belgium, France, and Spain revealed that strains of serotypes O15:K−:H−, O26:K−:H11, O103K−:H2, and O132:H2 were highly pathogenic for weaned rabbits (11, 39, 41).

Since no detailed reports exist on rabbit EPEC strains in Central Europe, we investigated the virulence genes of *E. coli* strains isolated from weaned rabbits in Hungary and the Czech Republic by using published PCR systems and by developing new PCR systems to detect genes of fimbrial adhesion factors of rabbit strains.

Sixty-six *E. coli* strains were isolated from caeca of 101 weaned rabbits: 40 diarrheal rabbits (22 strains) and 32 nondiarrheal rabbits (15 strains) obtained from one large rabbitry in Hungary and 29 diarrheal rabbits obtained from 17 rabbitries in the Czech Republic (29 strains).

The 66 *E. coli* strains were characterized by using different PCR assays for *E. coli* virulence genes, using previously pub-

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TABLE 1. PCR primers and reference *E. coli* strains used in this study

Primer pair	Gene	Product (bp)	Reference strain	Reference(s) for:	
				Primers	Control strains
B52/B53	*eaeA*	570	E2348/69	14	16
SK1/LP4	β-*eae*	2,287	RDEC-1	36	21
LT1/LT2	*lt*	275	EC263	2	30
Sta1/Sta2	*sta*	167	EC2173	2	31
STb1/Stb2	*stb*	138	EC2173	2	31
EAF1/EAF2	*EAF*	392	E2348/69	18	16
EP1/EP2	*Bfp*	747	E2348/69	20	16
B54/B55	stx_1	388	EDL933	14	43
B56/B57	stx_2	807	EDL933	14	43
B213/B214	*espD*	417	2348/69	19	16
Donne-280/281	*lifA*	2,225	E22	22	11
			E2348/69		16
CDT sense1/antisens1	*cdtB*	466	1404	48	16
CDT-sense2/antisens2					42
CNF-1-A/B	*cnf1*	526	J96	9	10
CNF-2-A/B	*cnf2*	526	1404	9	42
AF/R1-F/R	*afr1*	280	RDEC-1	Present study	21
AF/R2-F/R	*afr2*	518	E22	Present study	11
RalG-F/R	*ral*	577	83/39	Present study	38
155-F1/R1	*paa*	350	1390	5	4

lished primers and control strains (Table 1), except for the rabbit fimbriae AF/R1, AF/R2, and Ral, for which the following PCR primers and conditions were designed, in 30 cycles, as follows: AF/R1-F (5′TACCGTTACTGCGAAGACCT3′) and AF/R1-R (5′CGTGCTGTTAATCGCCACTA3′), 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s; AF/R2-F (5′AAGTTA GGGGACGCCATTAC3′) and AF/R2-R (5′CCAGGACTTA TTCTGACCAG3′), 94°C for 45 s, 57°C for 45 s, and 72°C for 45s; RalG-F (5′GATCTTTGGCAGTGGACAC3′) and RalG-R (5′CGGCAACAGTTCCTTTTGAA3′), 94°C for 45 s, 58°C for 60 s, and 72°C for 45 s. Cycles were finished by an extension time of 5 min at 72°C.

For colony dot blot hybridization for the major fimbrial subunit *ralG* genes, colonies of selected strains were grown overnight on Luria-Bertani agar plates, transferred to nitrocellulose membranes, lysed by alkali, and fixed as described by Mainil et al. (26). The *ralG*-specific PCR product was purified by using the QIAquick PCR purification kit (QIAGEN GmbH, Germany), and labeled with a digoxigenin ready-to-use labeling kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. Hybridization was carried out at 68°C, and the rest of the protocol was conducted according to the manufacturer's manual.

Phenotypic characteristics of the wild-type *E. coli* strains

TABLE 2. Characteristics of *E. coli* strains isolated from diarrheal and normal weaned rabbits

Serotype	Clinical state[e]	No. of strains[f]	No. *eae* positive	*eae* type	No. of strains positive for:					
					espD	*lifA*	*afr1*	*afr2*	*ral*	*paa*
O103:H2[a]	D	12	12	β	12	1		10		7
O157:H2[a,b]	D	4	4	β	4					4
O153:H7[a]	D	8	8	β	8	2	2		3	
	N	3	3	β	3				3	
O15:H?[c]	D	2	1	β	1					
	N	3	3	β	3				1	
O132:H2	D	4	4	β	4				4	1
O55:H7	D	1	1	β	1					1
O49:H2	D	1	1	β	1				1	1
	N	1	1	β	1				1	
O145:H?	D	1	1	β	1				1	1
Untypeable	D	4	4	β	4				1	1
	N	8								
Other defined[d]		14								
Total		66	43		43	3	2	10	15	16

[a] H antigens are determined for some but not for all the strains of the given serogroup.
[b] Some strains of the same serogroup are nonmotile.
[c] H?, H antigen could not be determined.
[d] O141 (2), O163 (2), O87 (2), O17, O4, O73, O77, O85 and OX (3) (the number of strains is indicated after the O type in parentheses).
[e] D, diarrheal rabbits; N, nondiarrheal rabbits.
[f] Eleven of the O103 strains and 7 of the O153 strains are from Czech rabbitries.

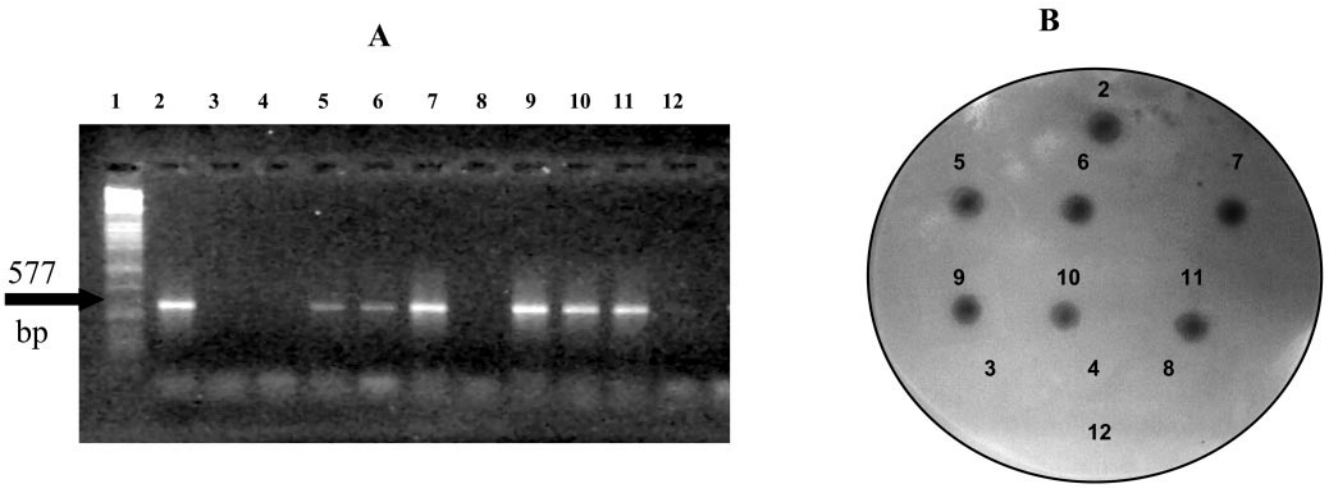

FIG. 1. Agarose gel electrophoresis of *ral* G-specific PCR products (A) and *ral*-specific colony hybridization (B). Samples in panels A and B were the same. Samples in gel electrophoresis: lane 1, molecular weight marker (λ phage DNA digested with PstI); lane 2, 83/39 *ral*-positive control strain; lanes 3 to 11, wild-type rabbit *E. coli* strains; lane 12, *E. coli* K-12 (DH5α), negative control. *E. coli* strains 5, 6, 7, 9, 10, and 11 were *ral* positive, and strains 3, 4, and 8 were *ral* negative.

included O typing (35), sugar fermentation, and hemolysin production (12). The HeLa cell adhesion assay was performed as described by De Rycke, et al. (15). AE activities of the rabbit EPEC strains were tested with ligated ileal loops of three weaned (6-week-old) rabbits as described by Moon et al. (29). Surgical anesthesia was provided by combined use of SBH-Ketamin (SelBruHa, Budapest, Hungary), Domitor (Pfizer), and Halothane (LECIVA, Prague, Czechoslovakia). Postsurgical comfort was provided under constant supervision by applications of Ketamin and Domitor for 12 to 14 h, followed by Nembutal (CEVA) euthanasia. AE lesions were identified by histopathology and electron microscopy (29).

Results of PCR testing of 66 *E. coli* strains isolated from weaned rabbits in both countries identified 43 strains with the intimin (*eae*) gene (36 from diarrheal rabbits and 7 from non-diarrheal rabbits), which were designated EPEC. The virulence attributes of the 43 rabbit EPEC strains are listed in Table 2. The F4(K88)/CS31A-related fimbrial genes *afr2* and *ral* were the two predominant adhesive fimbrial genes present. The presence of the *ral* gene was confirmed by colony hybridization with six randomly selected *ral*-positive strains, where they were hybridized with *ral*-specific probe, while none of the three *ral*-negative strains tested exhibited a positive signal (Fig. 1).

The most frequent serogroups of *eae*-positive strains were O103, O153, O15, O132, and O157 (Table 2). Regarding sorbitol (S) rhamnose (R) fermentation, of the 12 strains of the O103 serogroup, six were S^-/R^-, three were S^+/R^-, two were S^-/R^+, and one was S^+/R^+. Among the four strains belonging to O157, three strains were S^-/R^+ and one strain was S^-/R^-. The four strains belonging to O132:H2 were S^+/R^+. Representative strains of the most frequent EPEC serogroups with different fimbriae were tested for in vitro adhesion in the HeLa cell assay. All EPEC strains exhibited a diffuse adherence phenotype, defined as bacteria covering the whole cell surface rather than being limited to one or a few sites of the cell. The diffuse adherence phenotype was observed without any differences regarding the presence or type of fimbriae (Table 3). Adherence of bacteria and the presence of attaching and effacing lesions in ligated ileal loops of rabbits were observed with all of these representative strains (Table 3), which was confirmed by electron microscopy for both rabbit EPEC-infected samples taken for ultrastructural investigation (Fig. 2).

TABLE 3. Adherence phenotype of selected rabbit EPEC strains: production of AE lesion in rabbit intestine (in vivo) and adhesion to HeLa cells in vitro

Serogroup (*n*)[a]	Genetic attributes					Presence of AE lesion in rabbit intestine	Adherence to HeLa bacteria/cell[b]
	eae	*afr1*	*afr2*	*ral*	*paa*		
O153:H7 (1)	+	−	−	−	−	++	8.5 ± 0.45
O153:H7 (1)	+	+	−	−	−	+	14.3 ± 4.79
O103:H2 (1)	+	−	+	−	−	+	12.5 ± 1.99
O132:H2 (1)	+	−	−	+	−	+	12.7 ± 1.15
O157:H2 (1)	+	−	−	−	+	+	12.6 ± 0.65
E22 (1)	+	−	+	−	−	++	13.1 ± 0.55
E2348/69 (1)	+	−	−	−	−	NT[c]	16.3 ± 0.25
K12 (1)	−	−	−	−	−	−	0.07 ± 0.02

[a] *E. coli* strains: E22, O103:H2, rabbit EPEC; E2348/69, O127:H6:K1, human EPEC; K12, DH5α. *n,* no. of tested strains.
[b] Mean of at least three independent tests ± standard deviation.
[c] NT, not tested.

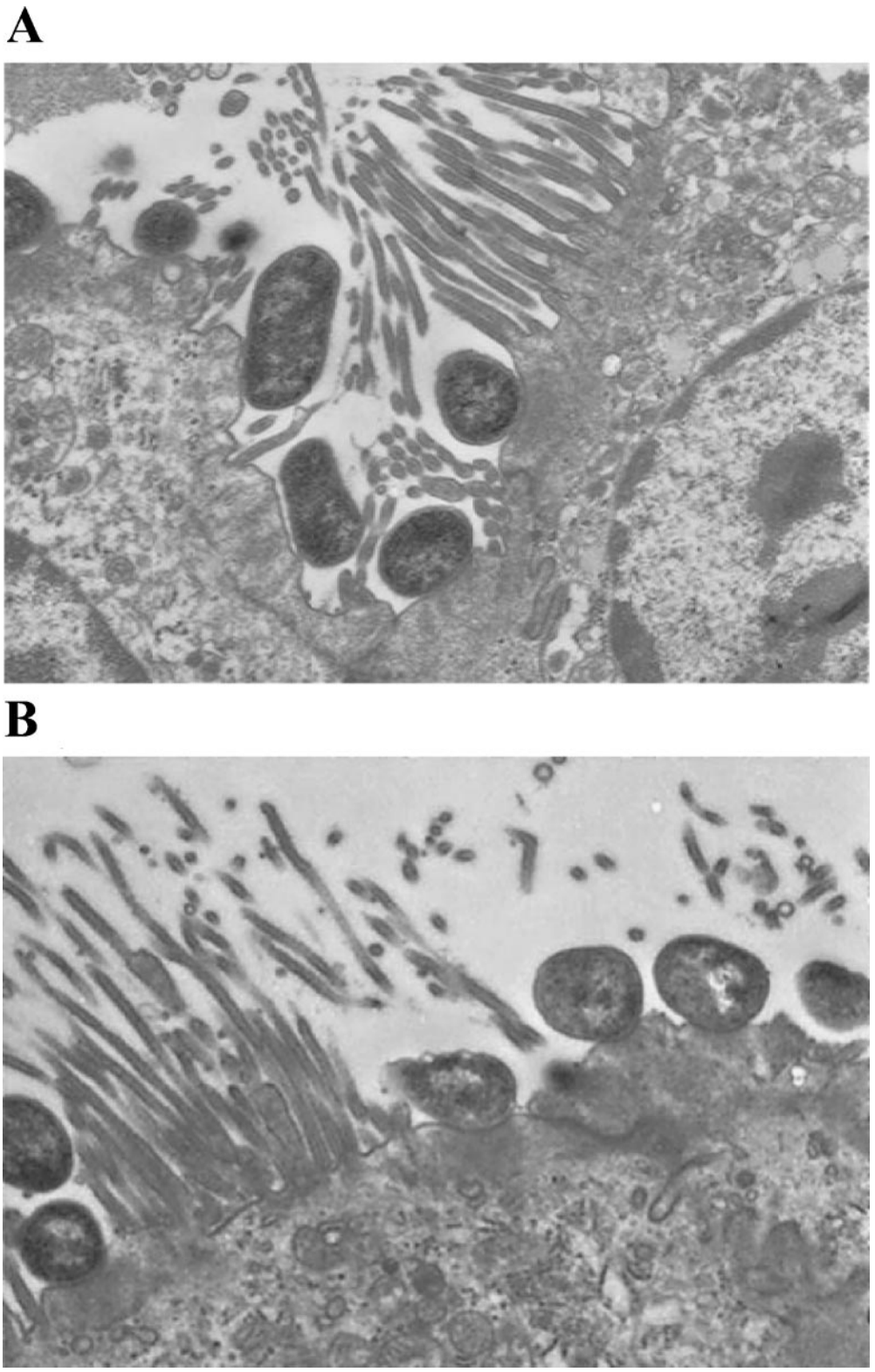

FIG. 2. Electron micrographs of surface epithelium from the ligated ileal loops of weaned rabbits infected with a rabbit O103:*rfa2* EPEC strain (A) or with a rabbit O153 EPEC strain without known rabbit or human fimbrial genes (B), illustrating intimate attachment of the bacteria to the epithelial cell membranes and effacement of the microvilli.

In this investigation we established that rabbit EPEC is the major type of pathogenic *E. coli* in rabbitries of Hungary and the Czech Republic, confirming reports from other countries (6, 11, 12, 18, 30, 32, 34, 37, 49). Furthermore, we detected the potential EPEC adhesins and established some peculiarities of rabbit EPEC in this region of Europe. First, we detected from rabbits in the Hungarian collection the classical human type EPEC O55:H7 bacteria, which have not been isolated from rabbits before (32). Interestingly, these bacteria did not harbor the genes of rabbit-specific fimbriae or *bfp* (specific to human EPEC) but possessed *paa*. Surprisingly, in this relatively small collection we also detected four strains of EPEC O157, of which one strain was O157:NM and three strains were O157:H2 (lacking rabbit fimbrial adhesins or *bfp* and all sorbitol negative). This seems to contrast to the reported isolation of O157 from rabbit (from Spain), where there was only 1 strain belonging to serogroup O157 out of 503 strains tested (8).

The EPEC fimbria called AF/R1, found in rabbits (13, 21), is a member of the family of type 1 fimbriae (class I adhesins): AfrA has 43 and 42% respective homologies with the major subunits of type 1 fimbriae (FimA) and Pap fimbriae (PapA), found to be associated with uropathogenic *E. coli* (50). AF/R1 is rarely detected in field isolates from rabbits (44). Using PCR primers designed for these studies, we found the presence of the *afr1* gene in two unrelated Czech EPEC strains belonging to O153, indicating that the presence of this type of fimbria also seems to be rare in Central Europe. However, we were successful in specifically detecting two recently described rabbit EPEC adhesin genes (*afr2* and *ral*) that are both related to F4(K88) fimbriae and to the nonfimbrial CS31A adhesin of porcine enterotoxigenic *E. coli* and bovine/porcine enterotoxigenic *E. coli*, respectively. The gene of adhesive factor/rabbit 2 (*afr2*) was detected in 11 strains of the O103 serogroup isolated from rabbits in both countries. The other recently identified adhesin, encoded by the rabbit EPEC adherence locus (*ral*) gene of an O15:H− EPEC (1), was detected here in 15 strains belonging to the O145, O153, O49, O15, and O132 serogroups. We also detected the porcine attaching and effacing-associated (*paa*) gene in 17 (38.8%) of the EPEC strains, with no association to any of the serogroups or to fimbrial types, while *lifA* was rare. Investigation of other, possibly cooperative adhesins (33) will be among aims of further studies.

In this study the representative *eae*-positive strains tested were able to adhere to HeLa cells and to produce AE lesions in the intestines of weaned rabbits independently of the type (AR/F1, AR/F2, or Ral) of their fimbrial adhesins. It is remarkable that several EPEC strains did not have rabbit-specific fimbrial adhesins. Two representatives of these strains (an O153:H7 strain and an O157:H2 strain) did adhere to HeLa cells in vitro and did produce AE lesions in vivo, like those with rabbit-specific fimbriae, suggesting the presence of further potential adhesins in some of these rabbit EPEC strains.

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